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14. ABSTRACT

Sporozoite infection of the liver is the first obligate step of the Plasmodium mammalian cycle. Inhibiting this step can block malaria at an early step. However, few anti-malarials target liver infection by sporozoites. Our goal is to find drugs that prevent or control liver infection. Development of such drugs will be facilitated by identification of parasite proteins required for liver infection. These proteins are potential drug targets for development of therapies that restrict Plasmodium liver infection. The aim of this Discovery award is to identify Plasmodium proteins that function in sporozoite invasion of hepatocytes and subsequent intrahepatic development.

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1. Introduction

Sporozoite infection of the liver is the first obligate step of the *Plasmodium* mammalian cycle. Inhibiting this step can block malaria at an early step. However, few anti-malarials target liver infection by sporozoites. Our goal is to find drugs that prevent or control liver infection. Development of such drugs will be facilitated by identification of parasite proteins required for liver infection. These proteins are potential drug targets for development of therapies that restrict Plasmodium liver infection. The aim of this Discovery award is to identify Plasmodium proteins that function in sporozoite invasion of hepatocytes and subsequent intrahepatic development.

2. Keywords

Plasmodium, sporozoites, liver infection, kinase, drugs, malaria

3. Accomplishments

- **What were the major goals of the project?**

Major goal of the project was to identify the target of a trisubstituted pyrolle (Tsp) molecule that is a potent inhibitor of Plasmodium sporozoite infection. The approach proposed in the project was to synthesize Tsp derivative appropriate for click-chemistry and to use these to identify parasite proteins that bind Tsp.

- **What was accomplished under these goals?**

Task 1: Synthesis and chemical characterization of Al-Tsp derivatives begins.

Two classes of Tsp derivatives (Al-Tsp) are appropriate for click chemistry (Fig. 1). Class I derivatives carry a photoaffinity azide group at various positions of the fluoroaryl ring and Class II carry a photoaffinity aryl azide groups in place of the pyridinyl ring. The goal of Task 1 was to synthesize Al-Tsp derivatives.

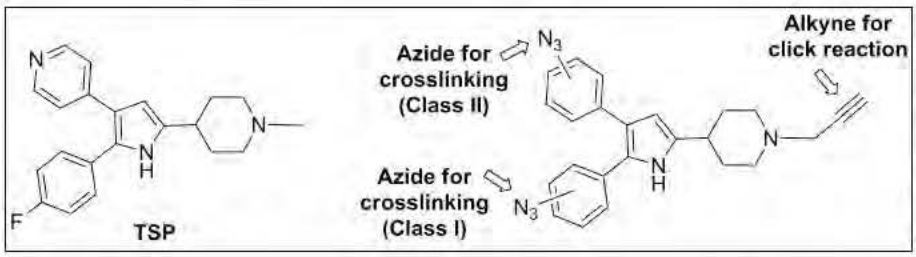

Figure 1: Examples of Tsp derivatives (Al-Tsp). *The crosslinking azide group will be placed at various positions of the fluoroaryl rings in Class I and pyridinyl ring in Class II derivatives.*

Guided by the previously employed methods to synthesize TSP and TSP derivatives, we demonstrated that we could append a propargyl group to the piperidine ring nitrogen and that this derivative retained activity. Thus the propargyl group would serve for click chemistry to isolate TSP derivatives cross-linked to target. Synthesis of aryl azide derivatives of TSP was then pursued, with the expectation to demonstrate activity was retained and that cross-linking could be achieved. Synthetic approaches were pursued to incorporate an azide group into TSP structure with each of the aryl rings. With either aryl ring, addition of an azide group or azide group precursor (nitro or aniline) to TSP or

late-stage TSP synthetic intermediate was not achieved. Thus TSP synthetic precursors having a nitro or protected aniline group were prepared, with the expectation that these precursors would be used in the synthesis of the requisite TSP derivatives, and the nitro or aniline groups would be converted to azide at intermediate or final stage of the synthesis. Ultimately, these substitutions to the precursor molecules afforded synthetic intermediates that failed to give desired products when using the methods previously employed to prepare TSP and TSP derivatives. Thus within the limited time-frame and budget of this study, the most direct synthetic routes with fewest synthetic steps to obtain the target derivatives failed. It is believed that the target azide derivatives are yet synthetically feasible.

However, with failure of the direct synthetic routes using modified intermediates and following the known TSP synthesis to give the target derivatives, future syntheses would require significant time, budget and effort to design and carry out a novel synthesis of TSP derivatives to incorporate the requisite azide groups for crosslinking.

Task 2: Begin HepG2 cell assays with different Al-Tsp derivatives.
Task 3: If AL-TSP identified in Year 1, optimize click chemistry conditions using reactive components. Further optimization carried out using bacterial extracts. If not, continue testing Al-Tsp derivatives. If all molecules are inactive, begin synthesis of Class II Tsp derivatives.

Task 2 and Task 3 were dependent on the successful synthesis of Al-Tsp derivatives in Task 1. Since Task 1 was unsuccessful at the end of year 1, Tasks 2 and 3 could not be undertaken as originally planned. Therefore, we modified our approach so that we could still fulfill the objective of identifying Tsp's target in sporozoites.

To fulfill the project's objective of identifying Tsp's target in sporozoites, we utilized an alternative approach of testing candidate proteins for their role in sporozoite invasion. Using two complementary strategies (a) specific small molecule inhibitors and (b) genetic mutants, we demonstrated that Plasmodium PKG is a target of Tsp during sporozoite invasion.

We used a transgenic *P. berghei* line expressing a HA-tagged, Tsp-resistant allele of PbPKG. The allele carries a substitution of the gatekeeper Thr residue (PKG T_{619}Q-HA) that prevents access of TSP to its binding pocket in P. berghei PKG. *P. berghei* parasites expressing PKG T_{619}Q are significantly less sensitive to inhibition by TSP but undergo normal schizogony, gametogenesis and sporozoite development [1]. We utilized these parasites to examine PbPKG's role in sporozoite infection.

PKG T_{619}Q sporozoites had lower infectivity compared to control sporozoites expressing the wildtype, HA-tagged Tsp-sensitive allele (PKG-HA) (Fig. 2A), suggesting that the PKG plays a role in sporozoite infection. To identify the steps of *Plasmodium's* liver cycle that require PKG, we quantified sporozoite invasion, and intracellular liver stages at different time-points. PKG T_{619}Q-HA sporozoites displayed an approximately two-fold decrease in the fraction of sporozoites that were intracellular 2h after addition to HepG2 cells, suggesting that PKG is required for sporozoite invasion (Fig. 2A). There was no further decrease in the number of intracellular liver stages at 24h post-infection (p.i) and 48h p.i (Fig. 2A). These data suggest that PKG is either not required for or its

decreased activity is sufficient for parasite remodeling in the parasitophorous vacuole, nuclear division or intra-vacuolar trophic growth.

To determine if PbPKG is essential for sporozoite infection of hepatocytes, we tested the sensitivity of PKG T_{619}Q-HA sporozoites to Tsp. Infection of HepG2 cells by PKG T_{619}Q-HA sporozoites was about 20-fold less sensitive to Tsp compared to control sporozoites expressing wildtype HA-tagged PKG (PKG-HA) (Fig. 2B). Higher doses of Tsp inhibited infection by both PKG-HA and T_{619}Q-HA sporozoites suggesting that Tsp could have additional targets secondary to PbPKG (data not shown). Because of these potential off-target effects, higher concentrations of Tsp were not used in subsequent experiments. The refractoriness of T_{619}Q-HA sporozoites to Tsp confirms that sporozoite infection of hepatocytes requires PbPKG, and that PbPKG is the primary target of Tsp in sporozoites. To verify that loss in sporozoite infectivity resulted from a block in sporozoite entry into cells, PKG-HA and PKG T_{619}Q-HA sporozoites were pre-treated with Tsp prior to addition to HepG2 cells. This brief treatment led to a dose-dependent decrease in the number of liver stages only in PKG-HA parasites supporting PbPKG's vital role in sporozoite invasion (Fig. 2B).

Merosome formation and/or release requires PKG. We previously showed that PKG cKO sporozoites do not form merosomes, suggesting that PKG is also required for parasite egress from hepatocytes [2]. These results were confirmed by testing the effect of TSP treatment on merosome formation by PKG-HA or T_{619}Q-HA sporozoites. Addition of TSP to HepG2 cells infected with PKG-HA sporozoites decreased the number of merosomes in the media in a dose-dependent manner (Fig. 2C). In contrast, the number of merosomes in the media of cultures infected with T_{619}Q-HA sporozoites was less sensitive to Tsp treatment. Both genetic and chemical inhibition confirm PKG's essential role in merosome formation and/or release.

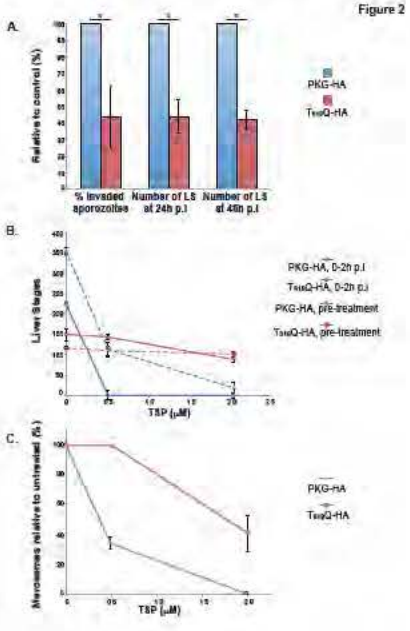

Figure 2: PbPKG is required for sporozoite invasion and merosome formation. *A) Decreased invasion by PKG T_{619}Q sporozoites results in reduced infectivity. PKG T_{619}Q and PKG-HA (control) sporozoites were added to HepG2 cells for 2h to determine the fraction of sporozoites that become intracellular. The number of infected HepG2 cells was determined at 24h and 48h p.i. B) Inhibition of PKG activity blocks sporozoite infectivity. HepG2 cells were infected with PKG T_{619}Q and PKG-HA sporozoites in the presence of TSP for 0-2h p.i (solid lines) or after pre-treatment of PKG T_{619}Q and PKG-HA sporozoites with TSP for 30min (dashed lines). Infected cells were quantified at 44h p.i. C) Inhibition of PKG activity prevents merosome formation. HepG2 cells infected with PKG-HA or T_{619}Q sporozoites were treated with TSP at 24-65h p.i. Number of merosomes released into the media was quantified at 66h p.i. Data was analyzed using two-tailed Mann-Whitney test, * $p < 0.05$.*

These and other results are reported in a manuscript currently under review:
Govindasamy K., Griscom B., Jebiwott, S., Khan R., Snyder M., Rana A., Li H., Bogyo M., Turk B., Brochet M., Billker O. and **Bhanot P.** Sporozoite invasion requires two kinases. *manuscript in review*

References Cited

1. Brochet M, Collins MO, Smith TK, Thompson E, Sebastian S, et al. (2014) Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca(2)(+) signals at key decision points in the life cycle of malaria parasites. PLoS Biol 12: e1001806.
2. Falae A, Combe A, Amaladoss A, Carvalho T, Menard R, et al. (2010) Role of Plasmodium berghei cGMP-dependent protein kinase in late liver stage development. J Biol Chem 285: 3282-3288.

- **What opportunities for training and professional development has the project provided?**

Nothing to report

- **How were the results disseminated to communities of interest?**

The P.I has presented a seminar at Rutgers New Jersey Medical School and is invited to speak at the Symposium on Global Health, Rutgers University, New Brunswick on June 30, 2015. In addition, a manuscript based on the results obtained thus far, with the PI as corresponding author, is under review by a peer-reviewed journal.

- **What do you plan to do during the next reporting period to accomplish the goals?**

In addition to demonstrating PbPKG's role in sporozoite invasion, we have demonstrated the role of another parasite kinase, CDPK4 in sporozoite invasion. We demonstrated that a small molecule inhibitor of CDPK4 (compound 1294) enhances the inhibitory effects of Tsp during sporozoite infection. Synergism in combinatorial therapy allows the same level of efficacy to be achieved with lower doses, improving safety and tolerability. Therefore, we are actively examining the possibility of synergistic action between Tsp and Compound 1294, in their inhibition of liver infection. In addition, ongoing studies are testing if PKG and CDPK4 are secondary targets of Compound 1294 and Tsp, respectively.

4. Impact

Our results are highly significant since they are the first demonstration of the role of cGMP signaling and Ca^{2+} signaling pathways in sporozoite invasion and liver stage development. These results strongly support further investigation of potential cross-talk between these pathways. Our data suggest that simultaneous inhibition of Plasmodium PKG and CDPK4 results in a potent block in sporozoite infection.

5. Changes/problems

- **Changes in approach and reasons for change**

Aim 1a of the proposal was to generate azide-linked derivatives of Tsp for click chemistry. This work was performed by Dr. Robert Kerns at the University of Iowa, as a subcontract. Despite attempts with multiple chemical routes, Az-Tsp could not be synthesized. Therefore, alternative approaches were considered for identifying potential protein targets of Tsp. Genetic and pharmacological approaches to examine potential candidate kinases were pursued in our laboratpru and proven to be highly successful.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report

- **Changes that had a significant impact on expenditures**

In July 2013, UMDNJ merged with Rutgers University. In July 2014, the Department of Microbiology & Molecular Genetics merged with the Department of Biochemistry. These head-to-head mergers resulted in a large number of administrative changes in grant administration. Due to delayed grant novation and numerous administrative changes, grant expenditure on salaries and supplies was slowed down. In addition, there were extensive lab relocations, including ours. Therefore, we have not completely finished the work on this grant. Results from Aim 1a demonstrated the need for genetic/ biochemical approaches to identify proteins required for sporozoite invasion. We have successfully identified 2 such proteins and are currently defining the potential cross-talk between them during sporozoite invasion. We are requesting a first-time No-Cost Extension until April 30, 2016 in order to finish the proposed work and continue building on our interesting findings.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report

6. Products

Journal publications (submitted): *Invasion of hepatocytes by Plasmodium sporozoites requires two kinases*. Govindaswamy, K., Griscom, B., Jebiwott, S., Khan, R., Snyder, M., Rana, A., Lou, H.J., Ojo, K.K., Van Voorhis, W.C, Brochet, M., Billker, O., Turk, B.E. and **Bhanot, P.**

7. Participants and other collaborating organizations

• What individuals have worked on the project?

Name:	*Purnima Bhanot*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.2*
Contribution to Project:	*Dr. Bhanot has supervised all aspects of this project and liased with the subcontractor Dr. Robert Kerns at the University of Iowa*
Funding Support:	

Name:	*Kavitha Govindasamy*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Govindasamy has worked on elucidating the actions of small molecule inhibitors on sporozoite infection*
Funding Support:	*The National Science Foundation*

• **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report. However, our request for a no-cost extension includes support for 100% effort of a postdoctoral fellow, Dr. Kumar to accelerate the completion of this project

7. What other organizations were involved as partners?

Nothing to report.

8. Special reporting requirements

Nothing to report.

9. Appendices N/A